

Cyanide-tolerant nitrile hydratases

BACKGROUND OF THE INVENTION

1. Field of the Invention

5 The invention relates to cyanide-tolerant nitrile hydratases especially from *Pseudomonas putida* or *Pseudomonas marginalis* strains which exhibit increased cyanide tolerance, to their use for preparing amides from nitriles in the presence of cyanides and to
10 polynucleotide sequences coding for this enzyme.

2. Description of the Related Art

The conversion of α -hydroxy nitriles (cyanohydrins) and α -amino nitriles into the corresponding amides using
15 nitrile hydratases opens up a novel variant for synthesizing α -hydroxy acids and α -amino acids because α -hydroxy amides and α -amino amides can be hydrolyzed in a simple manner (Process and catalysts for the production of methionine. Ponceblanc, Herve; Rossi, Jean-Christophe; Laval, Philip; Gros, Georges. (Rhone-Poulenc Animal Nutrition SA, Fr.), (WO 2001060789).
20 Alternatively, α -hydroxy amides can also be reacted with alkali metal or alkaline earth metal hydroxides to give the corresponding salts of the hydroxy acids. A particularly preferred reaction in this connection is
25 that of 4-methylthio- α -hydroxybutyramide (MHA amide) with calcium hydroxide, because calcium MHA can be employed directly as alternative form of product to methionine or MHA as feed additive.

30 However, α -hydroxy nitriles and α -amino nitriles readily decompose to aldehydes and hydrocyanic acid, and aldehydes, hydrocyanic acid and ammonia, respectively. The resulting hydrocyanic acid is a
35 strong inhibitor of almost all known nitrile hydratases with the exception of the nitrile hydratase from *Rhodococcus equi* XL-1, which shows the smallest loss of activity known to date at 20 mM cyanide (Production of

amides from nitriles by *Rhodococcus equi* cells having a cyanide resistant-nitrile hydratase. Nagasawa, Tohru; Matsuyama, Akinobu. (Daicel Chemical Industries, Ltd., Japan), (EP 1 266 962 A).

5 The low productivity of about 8 g of amide per g of dry biomass of resting cells, the long reaction time of 43 hours and the relatively low product concentration of 75 g/l lead to the search for improved nitrile hydratases.

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SUMMARY OF THE INVENTION

The aim of the invention described herein is therefore to provide a biocatalyst which is not subject to these restrictions. In addition, an even greater tolerance of
15 cyanide by the biocatalyst is advantageous, because α -hydroxy nitriles and α -amino nitriles are prepared, in order to ensure a rapid and complete reaction of the aldehyde, preferably with a 1-3% excess of hydrocyanic acid, part of which remains in the product. It is thus
20 possible for cyanide concentrations exceeding 20 mM to occur during the biotransformation. By-products and reagents such as amines employed as auxiliary bases must likewise not inhibit the nitrile hydratase activity.

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It is an object of the invention to provide nitrile hydratases which exhibit an increased stability to the cyanide ions present in the reaction solution during the conversion of nitriles to amides.

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DETAILED DESCRIPTION OF THE INVENTION

The invention relates to isolated polynucleotides, in particular from microorganisms of the genus *Pseudomonas*, which code for polypeptides having the
35 amino acid sequences which are 90 to 100% identical to the amino acid sequences comprised in the sequences SEQ

ID NO: 2, 3, 5, 7, 8, 10, where the polypeptides comprising the sequences SEQ ID NO: 2, 3, 5 or 7, 8, 10, together in each case have the activity of a cyanide-tolerant nitrile hydratase or form this nitrile
5 hydratase.

The polynucleotides are preferably derived from *Pseudomonas putida* or *Pseudomonas marginalis*.

The invention further relates to polynucleotides
10 selected from the group of

- a) polynucleotides comprising or consisting of the nucleotide sequences from SEQ ID NO: 1, 4, 6, 9 or nucleotide sequences complementary thereto,
15
- b) polynucleotides comprising nucleotide sequences which correspond to the sequences from a) within the scope of the degeneracy of the genetic code,
- 20 c) polynucleotides comprising nucleotide sequences as in a) which comprise functionally neutral sense mutations,
- 25 d) polynucleotides which hybridize with the complementary sequences from a) or c) under stringent conditions,

where the polynucleotides code for a cyanide-tolerant nitrile hydratase.
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The invention likewise relates to the polypeptides encoded by these polynucleotides and having the sequences SEQ ID NO: 2, 3, 5 or 7, 8, 10 with the activity of cyanide-tolerant nitrile hydratases from
35 microorganisms of the genus *Pseudomonas*, which may be present either enriched in the microorganisms or in

isolated form. SEQ ID NO: 2 and 7 code for the alpha subunits of the nitrile hydratases, SEQ ID NO: 3 and 8 for the beta subunits of the nitrile hydratases and SEQ ID NO: 5 and 10 for activator proteins whose
5 coexpression is essential for the activity of the nitrile hydratases (Nojiri et al., 1999, Journal of Biochemistry, 125: 696-704).

It is preferred according to the invention to use host
10 cells which have been transformed or transfected by the polynucleotides of the invention.

The host cells may belong to the eukaryotes or prokaryotes for which a stable expression system is
15 known, in particular

the host organisms preferably used are microorganisms for which there are expression systems, such as, for example, Pseudomonas, Pichia, various yeasts,
20 Saccharomyces, Aspergillus or the Streptomyces family, especially E. coli. Microorganisms of the genus Rhodococcus are likewise suitable.

Vector DNA can be introduced into eukaryotic or
25 prokaryotic cells by known techniques of transformation or transfection.

"Transformation", "transfection", conjugation" and "transduction" refer to procedures known in the state
30 of the arts for introducing foreign DNA.

The invention likewise relates to polynucleotides which consist essentially of one polynucleotide sequence, which are obtainable by screening by means of
35 hybridization of an appropriate gene library of Pseudomonas marginalis or Pseudomonas putida which comprises the complete gene or parts thereof, with a

probe which comprises the sequences of the polynucleotides of the invention from SEQ ID No: 1, 4 or 6, 9 or fragments thereof, and isolation of said polynucleotide sequence.

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Polynucleotides which comprise the sequences of the invention are suitable as hybridization probes for RNA, cDNA and DNA in order to isolate full-length nucleic acids and polynucleotides or genes which code for the proteins of the invention, or in order to isolate those nucleic acids and polynucleotides or genes which exhibit a great similarity of the sequences to those of the genes of the invention. They can likewise be attached as probe to so-called arrays, microarrays or DNA chips in order to detect and determine the corresponding polynucleotides or sequences derived therefrom, such as, for example, RNA or cDNA.

Polynucleotides which comprise the sequences of the invention are further suitable as primers with whose aid it is possible with the polymerase chain reaction (PCR) to prepare DNA of genes which code for the proteins of the invention.

Such oligonucleotides serving as probes or primers comprise at least 25 or 30, preferably at least 20, very particularly preferably at least 15, consecutive nucleotides. Oligonucleotides having a length of at least 40 or 50 nucleotides are likewise suitable. Also suitable where appropriate are oligonucleotides having a length of at least 100, 150, 200, 250 or 300 nucleotides.

"Isolated" means separated from its natural environment.

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"Polynucleotide" refers in general to polyribonucleotides and polydeoxyribonucleotides, possibilities being unmodified RNA or DNA or modified RNA or DNA.

5 The polynucleotides of the invention include polynucleotides of SEQ ID No: 1, 4, 6, 9 or fragments contained therein, and also those which are at least 90%, 93%, 95%, 97% or 99% identical to the polynucleotides of SEQ ID NO: 1, 4, 6, 9 or fragments
10 contained therein.

"Polynucleotides" mean peptides or proteins which comprise two or more amino acids connected by peptide linkages.

15 The polypeptides of the invention include polypeptides of sequences SEQ ID NO: 2, 3, 5, 7, 8, 10, and also those which are at least 90%, and particularly preferably at least 91%, 95%, 97% or 99% identical to
20 the polypeptides of sequences SEQ ID NO: 2, 3, 5, 7, 8, 10.

The DNA sequences obtained from the desired gene library can then be examined using known algorithms or
25 sequence analysis programs such as, for example, that of Staden (Nucleic Acids Research 14, 217-232 (1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

30 Coding DNA sequences which are derived from the sequences comprised in SEQ ID No. 1, 4, 6, 9 through the degeneracy of the genetic code are likewise an aspect of the invention. In the same way, DNA sequences
35 which hybridize with the sequences or parts thereof are an aspect of the invention. Also known to experts are conservative amino acid exchanges such as, for example,

exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. are functionally neutral. It is further
5 known that changes at the N or C terminus of a protein may not substantially impair or even stabilize its function. Details concerning this are to be found by a person skilled in the art inter alia in Ben-Bassat et al. (Journal of Bacteriology 169: 751-757 (1987)), in
10 O'Regan et al. (Gene 77: 237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3: 240-247 (1994)), in Hochuli et al. (Bio/Technology 6: 1321-1325 (1988)) and in well-known textbooks of genetics and molecular biology.

15 Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which are derived from SEQ ID NO: 1, 4, 6, 9 are an aspect of the invention. Such oligonucleotides typically have a length of at least 15 consecutive nucleotides, in
20 particular of 20, 30 or 40.

Instructions for the identification of DNA sequences by means of hybridization are to be found by a person skilled in the art inter alia in the handbook "The DIG
25 System Users Guide for Filter Hybridization" of Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260). The hybridization takes place under stringent conditions, meaning that
30 only hybrids in which probe and target sequence, i.e. the polynucleotides treated with the probe, are at least 90% identical are formed. It is known that the stringency of the hybridization including the washing steps is influenced or determined by variation in the
35 buffer composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out with relatively low stringency compared

with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

It is possible to employ for the hybridization reaction
5 for example a 5x SSC buffer at a temperature of about
50°C - 68°C. It is possible in this case also for
probes to hybridize with polynucleotides exhibiting
less than 70% identity to the sequence of the probe.
Such hybrids are less stable and are removed by washing
10 under stringent conditions. This can be achieved for
example by lowering the salt concentration to 2x SSC
and, where appropriate, subsequently 0.5x SSC (The DIG
System User's Guide for Filter Hybridisation,
Boehringer Mannheim, Mannheim, Germany, 1995), setting
15 a temperature of about 50°C - 68°C. It is possible
where appropriate to lower the salt concentration to a
0.1x SSC. It is possible by raising the hybridization
temperature stepwise in steps of about 1-2°C from 50°C
to 68°C to isolate polynucleotide fragments which have,
20 for example, at least 90% to 95% identity to the
sequence of the probe employed. Further instructions
for hybridization are obtainable on the market in the
form of so-called kits (e.g. DIG Easy Hyb from Roche
Diagnostics GmbH, Mannheim, Germany, Catalogue No.
25 1603558).

Instructions for the amplification of DNA sequences
with the aid of the polymerase chain reaction (PCR) are
to be found by a person skilled in the art inter alia
30 in the handbook by Gait: Oligonukleotide synthesis: A
Practical Approach (IRL Press, Oxford, UK, 1984) and in
Newton and Graham: PCR (Spektrum Akademischer Verlag,
Heidelberg, Germany, 1994).

35 The procedure is generally such that a gene which can
be expressed well is cloned into a vector with lower
copy number, genes with lower expression efficiency on

a vector with higher copy number and/or strong promoter. The host cells are transformed with these vectors in such a way that, compared with the initial organism, they comprise at least in each case one
5 additional copy of the nucleotide sequences coding for the formation of nitrile hydratase.

The transformed or recombinant microorganisms prepared in this way, especially of the genus *Pseudomonas*, are
10 likewise part of the invention.

It has been found that enhancement of the genes coding for the nitril hydratase of the invention and the helper protein P47K in microorganisms leads to an
15 increased production of the nitrile hydratase or else to an increased activity of the nitrile hydratase.

The term "enhancement" describes in this connection the increase in the intracellular activity of one or more
20 enzymes in a microorganism which are encoded by the appropriate DNA by, for example, increasing the copy number of the gene or of the genes, using a strong promoter or using a gene which codes for a corresponding enzyme with a high activity and, where
25 appropriate, combining these measures, compared with the non-recombinant initial organism.

To achieve overexpression it is possible to mutate the promoter and regulatory region or the ribosome binding
30 site which is located upstream of the structural gene. Expression cassettes incorporated upstream of the structural gene work in the same way. It is additionally possible to increase expression during the fermentative amino acid production by inducible
35 promoters. Expression is likewise improved by measures to extend the lifespan of the m-RNA.

In addition, the enzymatic activity is likewise enhanced by preventing degradation of the enzyme protein. The genes or gene constructs may be present either in plasmids with varying copy number or
5 integrated and amplified in the chromosome. A further alternative possibility is to achieve overexpression of the relevant genes by modifying the composition of the media and management of the culturing.

10 The invention likewise relates to

1) a process for the enzymatic preparation of amides from nitriles, which comprises the following steps:

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a) conversion of a compound which comprises a nitrile group or nitrile groups using a microbial enzyme which has nitrile hydratase activity and

20

b) removal of the amide formed, where

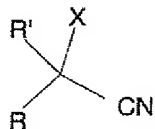
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c) a nitrile hydratase of the invention is employed for converting the nitrile into the amide. The remaining activity thereof after conversion of methacrylonitrile in the presence of 20 mM (mM = mmol/l) cyanide ions at 20°C after 30 min is preferably at least 90% of the remaining activity of the same enzyme when it
30 has been employed for the conversion in the absence of cyanide ions under conditions which are otherwise the same.

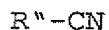
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2) a process according to 1), characterized in that the remaining activity after the conversion in the presence of 50 mM cyanide ions is at least 60%,

- 3) a process according to 1) or 2), characterized in that microorganisms producing and containing enzyme, or the lysate thereof, is/are employed.
- 5 4) a process according to 3), characterized in that resting cell of the microorganism is employed,
- 5) a process according to 1) or 2), characterized in that the purified enzyme is employed,
- 10 6) a process according to 1) to 5), characterized in that the enzyme is derived from microorganisms of the genus *Pseudomonas*, in particular *Pseudomonas putida* or *Pseudomonas marginalis*,
- 15 7) a process according to 6, characterized in that the enzyme is derived from microorganisms of the genus *Pseudomonas* deposited under the numbers DSM 16275 and DSM 16276, and which have amino acid sequences having the sequences SEQ ID NO: 2, 3, 5, 7, 8, 10,
- 20 8) a process according to one or more of points 1) to 7), characterized in that compounds of the general formulae
- 25



(I)



(II)

in which the meanings are:

X: OH, H, alkyl having 1 to 4 C atoms, NH₂
R: H, saturated alkyl radical having 1 to 12 C
atoms, branched or unbranched, optionally
NH₂-substituted
5 unsaturated alkyl radicals having a double
bond and 1 to 12 C atoms, branched or
unbranched, cycloalkyl groups having 3 to 6 C
atoms,
alkylene radicals substituted by alkylthio
10 groups, where alkyl here corresponds to a C₁
to C₃ radical,
and alkylene corresponds to a divalent C₃ to
C₈ radical,
R': H, if R is not H, alkyl having 1 to 3 C
15 atoms,
R'': mono- or binuclear unsaturated ring having 6
to 12 C atoms, optionally substituted by one
or two alkyl groups (C₁-C₃), Cl, Br, F,
monovalent alkyl nitrile radical having 1 to
20 6 C atoms,
are converted to the corresponding amides,

9) a process according to 8), characterized in that a
compound of the general formula (I) is converted
25 in the presence of hydrocyanic acid or a salt of
hydrocyanic acid,

10) a process according to 9), characterized in that
the conversion is carried out in the presence of
30 0.1 mol% cyanide to 3 mol% cyanide based on the
nitrile employed, preferably >2 to 3 mol%. This
corresponds at a final concentration of 1 mol to
30 mMol of cyanide at 3 mol%,

35 11) a process according to one or more of points 1) to
10), characterized in that methionine nitrile is
employed as nitrile,

- 12) a process according to one or more of points 1) to 10), characterized in that 2-hydroxy-4-methylthiobutyronitrile is employed as nitrile.

5

A reaction mixture like that obtained when hydrocyanic acid, 3-methylthiopropionaldehyde are reacted in the presence of an auxiliary base such as, for example, triethylamine according to the prior art is preferably employed.

10

It can advantageously be employed without purification.

15

This indicates the additional stability of the enzymes of the invention toward aldehydes and amines.

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- 13) A process in which 2-hydroxy-2-methylpropionitrile is employed as precursor for methacrylamide.

20

- 14) The invention is likewise directed to isolated and purified microorganisms of the genus *Pseudomonas*, deposited under the numbers DSM 16275 (MA32, *Pseudomonas marginalis*) and DSM 16276 (MA113, *Pseudomonas putida*), and

25

- 15) cyanide-tolerant nitrile hydratases isolated from the strains of the genus *Pseudomonas*, in particular from the strains of *Pseudomonas putida* and *Pseudomonas marginalis* deposited under the numbers DSM 16275 and DSM 16276.

30

The deposition took place on March 9, 2004, at the DSMZ, Deutsche Sammlung für Mikroorganismen und Zellkulturen in Brunswick, in accordance with the Budapest treaty.

35

The strains are particularly suitable for producing the enzymes of the invention.

- 5 "Isolated and purified microorganisms" relates to microorganisms which are present in a higher concentration than found naturally.

10 The invention likewise relates to a process for preparing the cyanide-tolerant nitrile hydratase described above, in which

- 15 a) a microorganism producing this nitrile hydratase, in particular of the genus *Pseudomonas marginalis* or *Pseudomonas putida*, is fermented under conditions with which the enzyme is formed in the microorganism, and
- 20 b) the cells are harvested at the earliest after the logarithmic growth phase has been completed.

Subsequently,

- 25 a) either the microorganism comprising the enzyme in the form of resting cells, where appropriate after increasing the permeability of the cell membrane, or
- 30 b) the lysate of the cells or
- c) the enzyme isolated from the cells of the microorganism using known measures

is employed for the conversion according to the invention of nitriles into amides.

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The nitrile hydratase may be either an enzyme generated with non-recombinant microorganisms or an enzyme generated recombinantly.

5 The invention additionally relates to processes for the recombinant preparation of the polypeptides of the invention, where a microorganism producing these polypeptides is cultivated, where appropriate expression of the relevant polynucleotides is induced,
10 and the enzymes are isolated where appropriate from the culture.

The process is generally one in which

15 a) microorganisms in particular of the genera *Pseudomonas marginalis* or *Pseudomonas putida* in which isolated polynucleotides from microorganisms of the family *Pseudomonas* which code for polypeptides having the amino acid sequences which
20 are 90 to 100% identical to the amino acid sequences comprising sequences in SEQ ID NO: 2, 3 and 5 or 7, 8 and 10, where the polypeptides in each case jointly have the activity of a cyanide-tolerant nitrile hydratase, enhanced, in
25 particular recombinantly overexpressed, are fermented,

b) the enzyme having nitrile hydratase activity is isolated where appropriate from these
30 microorganisms, or a protein fraction comprising this enzyme is prepared, and

c) the microorganism according to a) or the enzyme according to or the fraction comprising the latter
35 b) is transferred into a medium which comprises a compound comprising nitrile groups of the general formulae (I) and (II).

The culture medium used for the fermentation must comply in a suitable manner with the demands of the respective strains. Descriptions of culture media of various microorganisms are present in the handbook
5 "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

It is possible to use as carbon source sugars and
10 carbohydrates such as, for example, glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats such as, for example, soybean oil, sunflower oil, peanut oil and coconut fat, fatty acids such as, for example palmitic acid, stearic acid
15 and linoleic acid, alcohols such as, for example, glycerol and ethanol and organic acids such as, for example, acetic acid. These substances can be used singly or as mixture.

20 It is possible advantageously to use as nitrogen source organic nitriles or amides such as acetonitrile, acetamide, methacrylonitriles, methacrylamide, isobutyronitrile, isobutyramide or urea also in combination with other nitrogen-containing compounds
25 such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soybean flour and or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources may be used
30 singly or as mixture.

It is possible to use as phosphorus source phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-
35 containing salts. The culture medium must additionally comprise salts of metals, such as, for example, magnesium sulfate or iron sulfate, which are necessary

for growth. Finally, essential growth factors such as amino acids and vitamins can be employed in addition to the abovementioned substances. Said starting materials can be added to the culture in the form of a single
5 batch or be fed in during the cultivation in a suitable manner.

The pH of the culture is controlled in a suitable manner by employing basic compounds such as sodium
10 hydroxide, potassium hydroxide, ammonia or aqueous ammonia or acidic compounds such as phosphoric acid or sulfuric acid. Foaming can be controlled by employing antifoams such as, for example, fatty acid polyglycol esters. Aerobic conditions are maintained by
15 introducing oxygen or oxygen-containing gas mixtures such as, for example, air into the culture. The temperature of the culture is normally 10°C to 40°C and preferably 10°C to 30°C. The culture is continued until it has passed through the logarithmic growth phase.
20 This aim is normally achieved within 10 hours to 70 hours. Following this, the cells are preferably harvested, washed and taken up in a buffer as suspension at a pH of 6-9, in particular of 6.8 to 7.9. The cell concentration amounts to 1-25%, in particular
25 1.5 to 15% (wet weight/v). The permeability can be increased by physical or chemical methods, e.g. with toluene as described in Wilms et al., J. Biotechnol., Vol. 86 (2001), 19-30, so that the nitrile to be converted can penetrate through the cell wall and the
30 amide can emerge.

The following nitriles are preferably converted:

saturated mononitriles:
35 acetonitrile, propionitrile, butyronitrile, isobutyronitrile, valeronitrile, isovaleronitrile, capronitrile

saturated dinitriles:

malonitrile, succinonitrile, glutaronitrile, adiponitrile

5 aromatic unsubstituted and substituted mono- and dinitriles:

benzonitrile, 2,6-difluorobenzonitrile, phthalonitrile, isophthalonitrile, terephthalonitrile,

10 α -amino nitriles:

α -aminopropionitrile, α -aminomethylthiobutyronitrile, α -aminobutyronitrile, aminoacetonitrile, all nitriles derived from natural amino acids, α -amino-3,3-dimethylpropionitrile, α -amino-2,3-dimethylpropionitrile

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nitriles with carboxyl groups:

cyanoacetic acid

β -amino nitriles:

20 3-aminopropionitrile

unsaturated nitriles:

acrylonitrile, methacrylonitrile, allyl cyanide, crotononitrile

25

α -hydroxy nitriles:

α -hydroxy-n-propionitrile, α -hydroxy-n-butyronitrile, α -hydroxyisobutyronitrile, α -hydroxy-n-hexanonitrile, α -hydroxy-n-heptanonitrile, α -hydroxy-n-octanonitrile, 30 α,γ -dihydroxy- β,β -dimethylbutyronitrile, acrolein cyanohydrin, methacrylaldehyde cyanohydrin, 3-chlorolactonitrile, 4-methylthio- α -hydroxybutyronitrile and α -hydroxyphenylpropionitrile.

35 The concentration of the nitriles to be converted in the reaction solution is not limited to particular ranges.

In order to avoid inhibition of the enzymatic activity via the substrate, the concentration of the nitrile is generally maintained at 0.02 to 10 w/w%, in particular
5 0.1 to 2 w/w%, based on the amount of the biocatalyst as dry biomass. The substrate can be added as a whole at the start of the conversion or continuously or discontinuously during the conversion.

10 The dry weight is determined using the Moisture Analyser MA 45 (Sartorius).

If the solubility of the nitrile compound in the aqueous reaction system is too low, a solubilizer can
15 be added.

The reaction may, however, alternatively also be carried out in a water/organic solvent two-phase system.

20 When the cells of the microorganism are used as enzymatically active material, the amount of the cells employed is preferably 0.02 to 10 w/w% as dried biomass in relation to the amount of substrate.

25 It is also possible for the isolated enzyme to be immobilized by generally known techniques and then to be employed in this form.

30 The reaction is generally carried out at temperatures from -5°C to 50°C, in particular 0°C to 30°C, and for a time of from 0.1 to 100 hours.

The pH of the reaction mixture which is to be
35 maintained is not limited to particular values as long as the enzymatic activity is not impaired. After the

conversion, the amide formed can be removed from the reaction solution as known and be purified.

5 The invention likewise relates to a process in which the amide or the solution comprising the amide is separated for example from the cells of the biomass, and the amide is either hydrolyzed to the corresponding acid or converted with addition of alkali metal or alkaline earth metal hydroxides to the corresponding salts of the acids. MHA amide is preferably hydrolyzed with calcium hydroxide and the corresponding calcium salt is isolated.

Examples

15

Example 1

Culturing conditions

20 The precultures were grown in a volume of 5 ml in glass tubes, shaking at 30°C over the course of 24 h. 100 ml of the main culture were inoculated with 1 ml of the preculture and shaken in an Erlenmeyer flask with a total volume of 1000 ml at 25°C for 42 h.

25

Medium for the preculture (pH 7.0)	
K ₂ HPO ₄	7 g
KH ₂ PO ₄	3 g
Na citrate	0.5 g
Glycerol	2 g
FeSO ₄ * 7 H ₂ O	0.004 g
MgSO ₄ * 7 H ₂ O	0.1 g
Acetamide	2 g
Trace salt solution	0.1 ml
Demineralized water	Ad. 1000 ml

Medium for the main culture (pH 7.0)	
K ₂ HPO ₄	7 g
KH ₂ PO ₄	3 g
Sodium citrate	0.5 g
Glycerol	2 g
FeSO ₄ * 7 H ₂ O	0.004 g
MgSO ₄ * 7 H ₂ O	0.1 g
Acetamide	10 g
Trace salt solution	0.1 ml
Demineralized water	Ad. 1000 ml

Trace salt solution	
EDTA, Na ₂ * 2 H ₂ O	158 mg
Na ₂ MoO ₄ * 2 H ₂ O	4.7 mg
ZnSO ₄ * 7 H ₂ O	70 mg
MnSO ₄ * 4 H ₂ O	18 mg
FeSO ₄ * 7 H ₂ O	16 mg
CuSO ₄ * 5 H ₂ O	4.7 mg
CoSO ₄ * 6 H ₂ O	5.2 mg
Demineralized water	Ad. 1000 ml

Example 2

5 Isolation and identification of the microorganisms

The two strains MA32 and MA113 were selected by determining the nitrile hydratase activity of the resting cells in the presence of 2 mM potassium cyanide.

Properties of MA32:

Cell form	Rods
Width	0.6-0.8 µm
Length	1.5-3.0 µm

- 22 -

	Motility	+
	Flagella	polar > 1
	Gram reaction	-
5	Lysis by 3% KOH	+
	Aminopeptidase (Cerny)	+
	Oxidase	+
	Catalase	+
10	Growth at 41°C	-
	Substrate utilization	
	Adipate	-
	Citrate	+
15	Malate	+
	Phenylacetate	-
	D-Glucose	+
	Maltose	-
	Mannitol	+
20	Arabinose	+
	Mannose	+
	Trehalose	+
	Sorbitol	+
	Erythrol	+
25	Citraconate	+
	Inositol	+
	ADH	+
	Urease	-
30		
	Hydrolysis of gelatin	+
	Hydrolysis of esculin	+
	Levan from sucrose	+
35		
	Denitrification	+

Lecithinase +

Fluorescence +

Pyocyanin -

5

The profile of the cellular fatty acids is typical of Group I *Pseudomonas*

10 Analysis of a 484 bp-long segment of the 16S rRNA revealed a 100% agreement with the sequence of *Pseudomonas marginalis*

It was possible, taking account of all the data, to identify MA32 as *Pseudomonas marginalis*.

15

Properties of MA113:

Cell form Rods

Width 0.6-0.8 μm

20 Length 1.5-3.0 μm

Motility +

Flagella polar > 1

25 Gram reaction -

Lysis by 3% KOH +

Aminopeptidase (Cerny) +

Oxidase +

Catalase +

30

Growth at 41°C -

Substrate utilization

Adipate -

35 Citrate +

Malate +

Phenylacetate +

	D-Glucose	+
	Maltose	-
	Mannitol	-
	Arabinose	-
5	Mannose	-
	Trehalose	-
	Inositol	-
	β -Alanine	+
	α -Ketoglutarate	+
10	Benzylamine	+
	Hippurate	+
	Azelate	+
	D-mandelate	+
15	ADH	+
	Urease	-
	Hydrolysis of gelatin	-
	Hydrolysis of esculin	-
20	Levan from sucrose	-
	Denitrification	-
25	Lecithinase	-
	Fluorescence	+
	Pyocyanin	-
30	The profile of the cellular fatty acids is typical of Group I <i>Pseudomonas</i>	
	Analysis of a 476 bp-long segment of the 16S rRNA revealed a 100% agreement with the sequence of	
35	<i>Pseudomonas putida</i>	

It was possible, taking account of all the data, to identify MA113 as *Pseudomonas putida*.

Example 3

5

Determination of the enzymatic activity

The cells were grown as described in Example 1, removed from the culture medium by centrifugation and resuspended in standard buffer (50 mM potassium phosphate buffer of pH 7.5). 50 μ l of this cell suspension were added to 700 μ l of the standard buffer, and the reaction was started by adding 250 μ l of a 200 mM solution of the nitrile in standard buffer. The concentration of the cells in the cell suspension was in this case such that the nitrile was 5-30% converted after 10 min at 20°C. After 10 min at 20°C, the reaction was stopped by adding 20 μ l of 50% concentrated phosphoric acid, and the cells were removed by centrifugation.

HPLC analysis	
Column	Intersil ODS-3V (GL Sciences Inc.)
Mobile phase	Mixture of 10 mM potassium phosphate buffer of pH 2.3 and acetonitrile in the ratio 85:15 for methionine nitrile, MHA nitrile and acetone cyanohydrin, and 99:1 for all other substrates
Flow rate	1 ml/min
Detection	UV at 200 nm

The activity of one U is defined as the amount of enzyme which converts 1 μ mol of methacrylonitrile to the amide in one minute. If the acid was also produced in addition to the amide, one U was defined as the amount of enzyme which converts 1 μ mol of

25

methacrylonitrile into the amide and acid in one minute.

The relative activities of the strains MA32 and MA113
5 are depicted in figure 1 and in figure 2.

Example 4

Influence of cyanide on the activity of the nitrile
10 hydratase

50 μ l of a cell suspension prepared in analogy to
Example 3 were added to 700 μ l of the standard buffer
which comprised 0, 21.4, 53.6 and 107.1 mM potassium
15 cyanide (final concentration 0, 20, 50, 100 mM
cyanide). The reaction was started by adding 200 μ l of
a 200 mM solution of the nitrile in standard buffer
which in each case had the same cyanide concentration
as the remaining reaction solution. The concentration
20 of the cells in the cell suspension was in this case
such that the nitrile was 16% converted in the mixture
without cyanide after 10 min at 20°C. After 10 min at
20°C, the reaction was stopped by adding 20 μ l of 50%
concentrated phosphoric acid, and the conversion was
25 determined in analogy to Example 2.

The relative activities for the conversion of
methacrylonitrile as a function of the cyanide
concentration are shown in figure 3 and in figure 4.
30

Example 5

Conversion of acetone cyanohydrin with resting cells of
Pseudomonas marginalis MA32
35

Pseudomonas marginalis MA32 cells were grown and
centrifuged as described in Example 1. An amount of the

cells which comprised 1.16 g of dry biomass was diluted with 50 mM potassium phosphate buffer of pH 8.0 to a final volume of 50 ml. In addition, 0.02 mM of 2-methyl-1-propaneboronic acid was added to the reaction mixture. Freshly distilled acetone cyanohydrin was added continuously at 4°C with vigorous stirring at such a rate that the concentration did not exceed 5 g/l at any point during the reaction. The pH was kept constant at 7.5. The reaction was followed by HPLC as described in Example 3. After 140 min, 10.0 g of the nitrile had been completely converted into 10.7 g of amide and 1.4 g of acid.

The time course of the reaction achieved with the strain MA113 is depicted in figure 5.

Example 6

Conversion of crude MHA nitrile with resting cells of *Pseudomonas marginalis* MA32

Pseudomonas marginalis MA32 cells were grown and centrifuged as described in Example 1. An amount of the cells which comprised 0.34 g of dry biomass was diluted with 50 mM potassium phosphate buffer of pH 8.0 to a final volume of 70 ml. In addition, 0.02 mM 2-methyl-1-propaneboronic acid was added to the reaction mixture. The crude MHA nitrile was added continuously at 4°C with vigorous stirring at such a rate that the concentration did not exceed 10 g/l at any point during the reaction. The pH was kept constant at 8.0. The reaction was followed by HPLC as described in Example 3. After 510 min, 10.05 g of the nitrile had been completely converted into 11.13 g of amide and 0.31 g of acid. This corresponds to a final concentration of 139 g of amide per liter.

The MHA nitrile had been prepared directly from 3-methylthiopropionaldehyde and a slight excess of hydrocyanic acid. A 50 mM solution of this MHA nitrile in water contained 0.5 mM cyanide (Spektroquant®,
5 Merck).

The time course of the reaction achieved with the strain MA32 is depicted in figure 6.

10 Example 7

Cloning of the nitrile hydratase gene cluster from *Pseudomonas marginalis* MA 32 and construction of an expression vector

15

The gene cluster of the nitrile hydratase comprising an α subunit, β subunit and a nitrile hydratase activator protein whose coexpression is essential for the activity of the nitrile hydratase (Nojiri et al., 1999,
20 Journal of Biochemistry, 125: 696-704) was amplified by PCR using the primers 1F and 1R which introduced cleavage sites for the restriction enzymes NdeI and HindIII. The PCR product obtained in this way was ligated into a vector cut with NdeI and HindIII, with
25 the introduced genes being under the control of the rhamnose promoter. The expression vector produced in this way is called pKE31.

The restriction map is to be found in figure 7 and the
30 sequence in SEQ ID NO:1.

The expression plasmid was transformed into the strain *E. coli* DSM 14459 which had been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen
35 GmbH (DSMZ) on Aug. 22, 2001.

Primers:

1F	5'-CTC CAC CAT ATG AGT ACA GCT ACT TCA ACG -3'
1R	5'-CTT CAT AAG CTT CTA TCT CGG ATC AAA TGG-3'

1F: SEQ ID NO: 11

5 1R: SEQ ID NO: 12

The genes are located on the following segments of
SEQ ID NO: 1:

10 Gene of the α subunit: nt 25-609
Gene of the β subunit: nt 650-1312
Gene of the activator protein: nt 1309-2577

Example 8

15

Cloning of the nitrile hydratase gene cluster from
Pseudomonas putida MA113

20 The gene cluster of the nitrile hydratase consisting of
 α subunit, β subunit and a nitrile hydratase activator
protein whose coexpression is essential for the
activity of the nitrile hydratase (Nojiri et al., 1999,
Journal of Biochemistry, 125: 696-704) was amplified by
PCR using the primers 1F and 1R.

25

The sequence is to be found in SEQ ID NO: 6.

Primers:

2F	5'-ATG ACG GCA ACT TCA ACC CCT GGT G-3'
2R	5'-TCA GCT CCT GTC GGC AGT CG-3'

30

2F: SEQ ID NO: 13

2R: SEQ ID NO: 14

5 The genes are located on the following segments of
SEQ ID NO: 5:

Gene of the α subunit: nt 1-582

Gene of the β subunit: nt 624-1286

Gene of the activator protein: nt 1283-2360

10

Example 9

Heterologous expression of the nitrile hydratases from
Pseudomonas marginalis MA 32 in E. coli DSM 14459

15

E. coli DSM 14459 was deposited in connection with DE
101 55 928.

20 The cells transformed with pKE31 were grown in LB
medium (LB broth, Miller, VWR) which contained 2 mM
iron(III) citrate and 100 μ g/ml ampicillin at 37°C with
shaking. After 12-16 hours, an amount of the preculture
was transferred into a main culture such that the
latter had an OD600 of 0.1. The culture medium of the
25 main culture corresponded to that of the preculture but
additionally contained 2 g/l L-rhamnose. The cells were
harvested after cultivation at 30°C for 22 hours.

Example 10

30

Determination of the enzymatic activities

The culturing of the cells and the determination of the
activity were carried out as described in Example 9 and
35 Example 3.

The cells of the strain E. coli DSM 14459 transformed with the plasmid pKE31 had a specific activity of 17 U/mg of DBM.

5 Example 11

Determination of the enzymatic activities in the presence of 100 mM potassium cyanide

- 10 The culturing of the cells and the determination of the activities in the presence of 100 mM potassium cyanide were carried out as described in Example 9 and Example 4.
- 15 The cells of the strain E. coli DSM 14459 transformed with the plasmid pKE31 had a specific activity of 11 U/mg of DBM.

What is claimed is:

1. (Original) An isolated polynucleotide which codes for a polypeptide having the amino acid sequence which is 90 to 100% identical to the amino acid sequences contained in the sequences SEQ ID NO: 2, 3 and 5 or 7, 8 and 10.
2. (Original) The polynucleotide as claimed in claim 1, selected from the group:
 - a) polynucleotides comprising the nucleotide sequences SEQ ID NO: 1, 4, 6, 9 or nucleotide sequences complementary thereto,
 - b) polynucleotides comprising nucleotide sequences which correspond to the sequences from a) within the scope of the degeneracy of the genetic code,
 - c) polynucleotides comprising nucleotide sequences as in a) which comprise functionally neutral sense mutations,
 - d) polynucleotides which hybridize with the complementary sequences from a) under stringent conditions, where stringent conditions mean washing in 5XSSC at a temperature of from 50 to 65°C,where the polynucleotides code for a cyanide-tolerant nitrile hydratase.
3. (Original) A polypeptide comprising amino acid sequences which are 90 to 100% identical to the sequences to the sequences SEQ ID NO: 2, 3 and 5 or 7, 8 and 10.

4. (Original) The polypeptide having the activity of cyanide-tolerant nitrile hydratases as claimed in claim 3, whose remaining activity after conversion of methacrylonitrile in the presence of 20 mM (mM=mmol/l) cyanide ions at 20°C after 30 min is at least 90% of the remaining activity of the same enzyme when it has been categorized for the conversion in the absence of cyanide ions under conditions which are otherwise the same.
5. (Original) A probe or primer comprising at least 20 consecutive nucleotides from the sequences SEQ ID NO: 1, 4, 6, 9.
6. (Currently amended) A vector comprising a polynucleotide selected from the group consisting of:
 - (a) the polynucleotide of claim 1;
 - (b) a polynucleotide comprising the nucleotide sequences SEQ ID NO: 1, 4, 6, 9 or nucleotide sequences complementary thereto, where the polynucleotides code for a cyanide-tolerant nitrile hydratase;
 - (c) a polynucleotide comprising nucleotide sequences which correspond to the nucleotide sequences SEQ ID NO: 1, 4, 6, 9 or nucleotide sequences complementary thereto, within the scope of the degeneracy of the genetic code, where the polynucleotides code for a cyanide-tolerant nitrile hydratase;
 - (d) a polynucleotide comprising the nucleotide sequences SEQ ID NO: 1, 4, 6, 9 or nucleotide sequences complementary thereto, which comprise functionally neutral sense mutations, where the polynucleotides code for a cyanide-tolerant nitrile hydratase;
 - (e) a polynucleotide which hybridizes with the complementary sequences of nucleotide sequences

SEQ ID NO: 1, 4, 6, or 9 under stringent conditions, where stringent conditions mean washing in 5XSSC at a temperature of from 50 to 65°C, where the polynucleotides code for a cyanide-tolerant nitrile hydratase.

7. (Currently amended) A host cell transformed or transfected by the introduction of a polynucleotide selected from the group consisting of:
- (a) the polynucleotide of claim 1;
 - (b) a polynucleotide comprising the nucleotide sequences SEQ ID NO: 1, 4, 6, 9 or nucleotide sequences complementary thereto, where the polynucleotides code for a cyanide-tolerant nitrile hydratase;
 - (c) a polynucleotide comprising nucleotide sequences which correspond to the nucleotide sequences SEQ ID NO: 1, 4, 6, 9 or nucleotide sequences complementary thereto, within the scope of the degeneracy of the genetic code, where the polynucleotides code for a cyanide-tolerant nitrile hydratase;
 - (d) a polynucleotide comprising the nucleotide sequences SEQ ID NO: 1, 4, 6, 9 or nucleotide sequences complementary thereto, which comprise functionally neutral sense mutations, where the polynucleotides code for a cyanide-tolerant nitrile hydratase;
 - (e) a polynucleotide which hybridizes with the complementary sequences of nucleotide sequences SEQ ID NO: 1, 4, 6, or 9 under stringent conditions, where stringent conditions mean washing in 5XSSC at a temperature of from 50 to 65°C, where the polynucleotides code for a cyanide-tolerant nitrile hydratase
- or a vector comprising the polynucleotide.

8. (Original) A host cell transformed by the introduction of a vector as claimed in claim 6.

9. (Currently amended) A process for the enzymatic preparation of amides from nitriles, which comprises the following steps:

- a) conversion of a compound comprising nitrile groups using a microbial enzyme (polypeptide) which has nitrile hydratase activity and
- b) removal of the amide formed,

employing a cyanide-tolerant nitrile hydratase selected from the group consisting of:

- (1) the polypeptide of claim 3;
- (2) a polypeptide having the activity of cyanide-tolerant nitrile hydratases comprising amino acid sequences which are 90 to 100% identical to the sequences to the sequences SEQ ID NO: 2, 3 and 5 or 7, 8 and 10, whose remaining activity after conversion of methacrylonitrile in the presence of 20 mM (mM=mmol/l) cyanide ions at 20°C after 30 min is at least 90% of the remaining activity of the same enzyme when it has been categorized for the conversion in the absence of cyanide ions under conditions which are otherwise the same,

for the conversion of the nitrile to the amide.

10. (Currently amended) The process as claimed in claim 9, wherein a host cell transformed or transfected by the introduction of a polynucleotide selected from the group consisting of:

- (a) a polynucleotide which codes for a polypeptide having the amino acid sequence which is 90 to 100% identical to the amino acid sequences contained in

the sequences SEQ ID NO: 2, 3 and 5 or 7, 8 and 10;

(b) a polynucleotide comprising the nucleotide sequences SEQ ID NO: 1, 4, 6, 9 or nucleotide sequences complementary thereto, where the polynucleotides code for a cyanide-tolerant nitrile hydratase;

(c) a polynucleotide comprising nucleotide sequences which correspond to the nucleotide sequences SEQ ID NO: 1, 4, 6, 9 or nucleotide sequences complementary thereto, within the scope of the degeneracy of the genetic code, where the polynucleotides code for a cyanide-tolerant nitrile hydratase;

(d) a polynucleotide comprising the nucleotide sequences SEQ ID NO: 1, 4, 6, 9 or nucleotide sequences complementary thereto, which comprise functionally neutral sense mutations, where the polynucleotides code for a cyanide-tolerant nitrile hydratase;

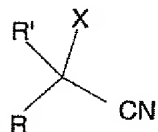
(e) a polynucleotide which hybridizes with the complementary sequences of nucleotide sequences SEQ ID NO: 1, 4, 6, or 9 under stringent conditions, where stringent conditions mean washing in 5XSSC at a temperature of from 50 to 65°C, where the polynucleotides code for a cyanide-tolerant nitrile hydratase

or a vector comprising the polynucleotide, or the lysate thereof, is employed.

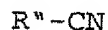
11. (Currently amended) The process as claimed in claim 10, wherein resting cells of the microorganism are employed.

12. (Currently amended) The process as claimed in claim 9, wherein a purified nitrile hydratase is employed.

13. (Currently amended) The process as claimed in claim 9, wherein the enzyme is derived from microorganisms of the genus *Pseudomonas*.
14. (Currently amended) The process as claimed in claim 13, wherein the enzyme is derived from employed microorganisms of the species *Pseudomonas putida* or *Pseudomonas marginalis*.
15. (Currently amended) The process as claimed in claim 14, wherein the employed microorganisms are deposited under the numbers DSM 16275 and DSM 16276.
16. (Currently amended) The process as claimed in claim 9, wherein compounds of the general formula



(I)



(II)

where

X is OH, H, alkyl, or NH₂;

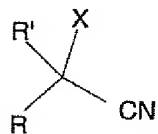
R is H, saturated alkyl radical having 1 to 12 C atoms, branched or unbranched, optionally NH₂-substituted, unsaturated alkyl radicals having a double bond and 1 to 12 C atoms, branched or unbranched, cycloalkyl groups having 3 to 6 C atoms, or alkylene radicals substituted by alkylthio groups, where alkyl here corresponds to a C₁ to C₃ radical, and alkylene corresponds to a divalent C₃ to C₈ radical,

R' is H, or an alkyl having 1 to 3 C atoms,
R'' is a mono- or binuclear unsaturated ring having
6 to 12 C atoms, optionally substituted by
one or two alkyl groups (C₁-C₃), Cl, Br, F, or
an alkyl nitrile radical having 1 to 6 C
atoms,
are converted to the corresponding amides.

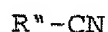
17. (Currently amended) The process as claimed in claim 16, wherein a compound of the general formula (I) is converted in the presence of hydrocyanic acid or a salt of hydrocyanic acid.
18. (Currently amended) The process as claimed in claim 17, wherein the conversion is carried out in the presence of an initial concentration of more than 0.5 mol% cyanide to 3 mol% cyanide, based on the nitrile employed.
19. (Currently amended) The process as claimed in claim 9, wherein 2-amino-4-methylthiobutyronitrile is employed as nitrile.
20. (Currently amended) The process as claimed in claim 9, wherein 2-hydroxy-4-methylthiobutyronitrile, where appropriate present in the reaction mixture from the preparation of this nitrile, is employed as nitrile.
21. (Currently amended) The process as claimed in claim 9, wherein 2-hydroxy-2-methylpropionitrile is employed as nitrile.
22. (Currently amended) The process as claimed in claim 9, wherein the amide or the solution comprising the amide is separated from the cells of the biomass, and the amide is hydrolyzed to the corresponding acid.

23. (Currently amended) The process as claimed in claim 9, wherein the amide or the solution comprising the amide is separated from the cells of the biomass, and the amide is hydrolyzed with alkali metal or alkaline earth metal hydroxides to the salts of the corresponding carboxylic acids.
24. (Currently amended) The process as claimed in claim 23, wherein MHA amide is hydrolyzed with calcium hydroxide, and the calcium salt is obtained.
25. (Currently amended) The process as claimed in claim 9, where
- a) microorganisms of the genus *Pseudomonas* in which isolated polynucleotides which code for polypeptides having the amino acid sequences which are 90 to 100% identical to the amino acid sequences comprised in the sequences with the sequences SEQ ID NO: 2, 3, 5, 7, 8, 10, where the polypeptides have the activity of a cyanide-tolerant nitrile hydratase, enhanced, in particular recombinantly overexpressed, are fermented,
 - b) the enzyme produced recombinantly and having nitrile hydratase activity is isolated where appropriate from these microorganisms, or a protein fraction comprising this enzyme is prepared, and
 - c) the microorganisms according to a) or the enzyme or the fraction comprising the latter according to b) is transferred into a medium which comprises a compound comprising nitrile groups of the general formulae (I) and (II).

26. (Currently amended) The process as claimed in claim 10, wherein compounds of the general formula



(I)



(II)

where

X is OH, H, alkyl, or NH₂;

R is H, saturated alkyl radical having 1 to 12 C atoms, branched or unbranched, optionally NH₂-substituted, unsaturated alkyl radicals having a double bond and 1 to 12 C atoms, branched or unbranched, cycloalkyl groups having 3 to 6 C atoms, or alkylene radicals substituted by alkylthio groups, where alkyl here corresponds to a C₁ to C₃ radical, and alkylene corresponds to a divalent C₃ to C₈ radical,

R' is H, or an alkyl having 1 to 3 C atoms,

R'' is a mono- or binuclear unsaturated ring having 6 to 12 C atoms, optionally substituted by one or two alkyl groups (C₁-C₃), Cl, Br, F, or an alkyl nitrile radical having 1 to 6 C atoms,

are converted to the corresponding amides.

27. (Original) A microorganism of the genus *Pseudomonas* deposited under the number DSM 16275 or DSM 16276.

28. (Original) A cyanide-tolerant nitrile hydratase isolated from the strains of the genus *Pseudomonas* deposited under the numbers DSM 16275 and DSM 16276.